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Genome-wide CRISPR-Cas9 screen

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An abbreviated version of this protocol was published in Science in Jun 2021

The lysosomal Rag-Ragulator complex licenses RIPK1– and caspase-8–mediated pyroptosis by Yersinia

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Detailed protocol

Genome-wide CRISPR-Cas9 screen:

1, Seed Cas9 stably expressed iBMDMs at 2×10^6 cells/dish in DMEM medium without antibiotics in 10 cm dish (30-35 dishes);
2, Infect the cells with lentivirus-containing sgRNA library at a multiplicity of infection (MOI) of 0.3 in DMEM medium containing 1 ng/μl polybrene (without antibiotics);
3, Change the medium at 24 h after infection;
4, Sixty hours later, cells were treated with 5 ng/μl puromycin to remove uninfected cells;
5, Change the medium every 24 h, and keep puromycin treatment for three days;
6, Six days after puromycin treatment, the transduced cells were seeded in 40 × 10 cm dishes (8×10^6 cells/dish) and treated with 10 ng/ml LPS plus 125 nM 5z7 for 6 hours (3×10^8 transduced cells were collected as the control sample);
7, Three days later, the surviving cells were reseeded and treated with 10 ng/ml LPS plus 250 nM 5z7 overnight;
8, Two days later, surviving cells were again treated with 20 ng/ml LPS plus 250 nM 5z7 overnight before surviving cells were harvested.

DNA extraction:

1, Surviving cells and control sample were lysed in the SNET buffer (20 mM Tris-HCl pH 8.0, 5 mM EDTA, 400 mM NaCl, 1% SDS and 400 mg/ml Proteinase K), 3×10^8 cell/5 ml buffer;
2, Incubate at 65°C, 2-3 h, until the sample is clear;
3, Add an equal volume of PCI (phenol/chloroform/isoamyl alcohol, 25:24:1, pH>7.8), mix and microcentrifuge 10 min at 12,000 rpm at room temperature;
4, Carefully transfer the top (aqueous) phase to a new tube;
5, repeat steps 3-4;
6, Add an equal volume of CI (chloroform/isoamyl alcohol 24:1), mix and centrifuge for 10 min at 12,000 rpm;
7, Carefully transfer the top (aqueous) phase to a new tube;
8, Add 2.5 vol of ice-cold 100% ethanol. Mix and place in -70°C overnight;
9, Spin 20 min at 14,000 rpm at 4°C and remove the supernatant;
10, Carefully add 1 ml cold 70% ethanol to wash the DNA pellets;
11, Spin 10 min at 12,000 rpm at 4°C and remove the supernatant;
12, Air dry the pellet at room temperature;
13, Dissolve the DNA in H_2O (0.5 μg/μl). Larger quantities of DNA may require vortexing and brief heating (5 min at 65 °C) to resuspend.

Next generation sequencing:

Genomic DNAs were amplified by two step PCR using the 2× Hieff Canace Gold PCR Master Mix:
1, Set up PCR reactions with 10 μg of genomic DNA in 100 μL final volume using 50 μL of 2× Hieff Canace Gold PCR Master Mix, and 2 μL each of 10 μM primers (Forward primer: 5'-AAT GGA CTA TCA TAT GCT TAC CGT AAC TTG AAA GTA TTT CG-3', Reverse primer: 5'-TCT ACT ATT CTT TCC CCT GCA CTG T-3');
2, Perform as many PCR replicates per sample as needed to maintain desired library coverage. For control sample, 30 PCR replicates were necessary, while 3 PCR replicates were sufficient for screening samples;
3, The following thermocycler conditions should be used: 98 °C for 30 S, 18×(98 °C for 10 s, 58 °C for 20 s, 72 °C for 25 s), 72 °C for 5 min;
4, Pool all primary PCR products for each sample.
5, Set up secondary PCR reactions using 100 μL final volume, 50 μL of 2× Hieff Canace Gold PCR Master Mix, 10 μL of the pooled primary PCR product and 2 μL each of 10 μM Illumina sequencing primers(1), 7 PCR replicates for each sample;
6, The following thermocycler conditions should be used: 98 °C for 30 S, 20×(98 °C for 10 s, 63 °C for 20 s, 72 °C for 10 s), 72 °C for 5 min;
7, Load samples on a 2% agarose Gel, and isolate the secondary PCR product at 260-270 bp;
8, Proceed with Next generation sequencing on an Illumina HiSeq 2500 sequencer.

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